Positive Interactions between Human Interferon and Cyclophosphamide or Adriamycin in a Human Tumor Model System

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ABSTRACT

Human lymphoblastoid interferon strongly increased the antitumor activity of suboptimal doses of two commonly used anticancer drugs, cyclophosphamide and Adriamycin, on a human breast tumor xenograft growing in nude mice. A combination of human lymphoblastoid interferon with either of these agents caused regression and in some cases total disappearance of tumors at doses of drug and interferon that, used singly, were capable only of inhibiting tumor growth. The combined therapy also resulted in a greatly increased survival. Studies with interferon and cyclophosphamide indicated that the antitumor activity was greatest when the two agents were administered simultaneously rather than sequentially.

INTRODUCTION

It is now well established that IFNs2 have antitumor activity in both animals and humans, but in many tumors IFN therapy alone is not sufficient to cause complete remission of established disease (13, 15, 16, 21). Two other possible roles for IFNs in human cancer would be as adjuvant therapy when the tumor load has been reduced by other means or as part of a combination between IFNs and agents such as chemotherapeutic drugs which have different mechanisms of action. Most chemotherapeutic drugs are predominantly cytotoxic, whereas IFNs appear to be cytostatic agents (4, 20), and there is increasing evidence that IFNs act not only directly on tumor cells (1) but also indirectly on host responses to the tumor (5, 11). We have previously reported that human IFN inhibited the development of human breast cancer xenografts in nude mice (3). More recently, we have shown that human IFN inhibited the growth of established breast cancer xenografts but did not cause any tumor regression (1). Here we describe the therapeutic potential of combinations of human IFN-α with 2 widely used drugs, CY and ADR, in this model system.

MATERIALS AND METHODS

Mice. Female nu/nu mice of mixed genetic background were bred by A. Sebesteny and J. Menzies (ICRF Laboratories, Mill Hill, United Kingdom) and maintained as described previously (1) except that for experiments 6- to 8-week-old specific-pathogen-free mice were used.

Tumor. The tumor used in all these experiments was derived from a mucoid carcinoma of the breast in an untreated postmenopausal woman. The tumor was established and passaged solely in nude mice and has a human karyotype. Details of its morphology and growth characteristics are given elsewhere (1). The tumor was used between passages 15 and 21 in the nude mouse.

Experimental Procedure. The tumor was excised and chopped with scissors until a fine suspension was obtained, and 1 ml of phosphate-buffered saline was added to ease implantation. Tumor suspension (0.05 ml) was inoculated ventrally s.c. into each mouse, and the mice were fed terramycin powder (2%) in their drinking water for the next week to prevent any infection at the site of implant. The tumors were allowed to grow for 2 to 3 weeks until the 2 largest diameters measured by calipers were between 0.3 and 0.9 cm. Therapy then commenced. The mice were given IFN s.c. at a site distant from the tumors in a volume of 0.2 ml and the ADR or CY i.p. in a volume of 0.05 to 0.20 ml. Tumor growth was assessed weekly by measuring the 2 largest diameters at right angles to each other, and the tumor size index as shown in the charts was a multiplication of these 2 measurements. Mice were killed when their tumor size index was between 2 and 4.

IFN and Drugs. Several batches of human IFN-α derived from Namalwa cells, HuIFN-α(Ly), prepared as reported (8), were used. The specific activity ranged from 1.17 to $2.20 \times 10^5$ units/mg protein. Prior to the experiment, IFN samples were assayed on at least 3 occasions in a viral RNA synthesis inhibition assay using WISH cells (Flow Laboratories, Irvine, United Kingdom) challenged with Semliki Forest virus. The assay was calibrated against human IFN Research Standard 69/19 (obtained from the National Institute of Biological Standards and Controls, Hampstead, London, United Kingdom). After correction was made for loss of activity in one freeze-thaw cycle, the IFN was diluted in phosphate-buffered saline plus bovine serum albumin (3 mg/ml; Sigma Chemical Co., Dorset, United Kingdom) and frozen down in single-dose aliquots. CY (Endoxana; W. B. Pharmaceuticals, Ltd., Bracknell, United Kingdom) and ADR (Farmitalia Carlo Erba, Ltd., Barnet, United Kingdom) were freshly diluted to the appropriate dose at each time point.

Drug and IFN doses in the mice were calculated to be equivalent to or lower than those tolerated in humans according to the surface area dosage conversion factors of Freireich et al. (9). Thus, a dose of $2 \times 10^6$ units IFN/25 g mouse (i.e., $80 \times 10^3$ units/kg) was equivalent to $6.7 \times 10^2$ units/kg or $20 \times 10^3$ units/sq m in humans. A dose of 0.25 mg CY/25 g mouse was equivalent to 32 mg/sq m in humans, and 0.04 mg ADR/25 g mouse was equivalent to 5.2 mg/sq m.

Peripheral Blood Cell Counts. At intervals throughout the experiment, approximately 20 μl of blood were collected from the tail vein of each mouse into a heparinized tube. This was diluted 1/10 in 4% acetic acid solution to lyse the RBC, and the WBC were counted under a phase-contrast microscope.

RESULTS

Effects on Breast Cancer Xenografts of Human Namalwa IFN, CY, or ADR, Given as Single Agents. Chart 1 shows that HuIFN-α(Ly), CY, and ADR all inhibited the growth of established human breast tumors in a dose-dependent fashion. The highest dose of CY and ADR in these experiments was the maximally tolerated dose, with 2 or more mice dying either during the therapy (see Chart 1B) or shortly afterwards. In Chart 1A, the differences between control tumor size index and that of the CY treatments over 0.5 mg were statistically significant as assessed by the Student t test (one-tailed test) ($p < 0.001$). In Chart 1B, the 0.04-mg-ADR group was statistically significant from the
IFN and Chemotherapy Combinations

Chart 1. Inhibitory effects of CY, ADR, and HulFN-α(Ly) on the growth of a human breast cancer xenograft. Each experimental group contained 4 mice. A, effect of CY, given once weekly i.p., 3 weeks in every 4. • control; A, CY, 0.1 mg; O, CY, 0.5 mg; ©, CY, 1.5 mg; 8, CY, 3.0 mg. B, effect of ADR, given once weekly i.p. • control; ©, ADR, 0.01 mg; 8, ADR, 0.02 mg; △, ADR, 0.04 mg; Δ, ADR, 0.08 mg. 1, all mice died during therapy of drug-induced intestinal toxicity. C, effect of IFN given s.c. daily. • control; 8, HulFN-α(Ly), 2 x 10^4 units; ©, HulFN-α(Ly), 5 x 10^4 units; ©, HulFN-α(Ly), 2 x 10^5 units.

Schart 2. IFN-CY combination therapy. CY was given i.p. 3 weeks in every 4; HulFN-α(Ly) was given s.c. daily. Each experimental group contained 4 mice. A. • control; △, CY, 0.25 mg; ©, HulFN-α(Ly), 2 x 10^4 units; □, HulFN-α(Ly), 2 x 10^5 units, plus CY, 0.25 mg. B, • control; △, CY, 0.25 mg; ©, HulFN-α(Ly), 5 x 10^4 units; □, HulFN-α(Ly), 5 x 10^5 units, plus CY, 0.25 mg. C, • control; △, CY, 0.25 mg; ©, HulFN-α(Ly), 2 x 10^4 units; □, HulFN-α(Ly), 2 x 10^5 units, plus CY, 0.25 mg.

control at p < 0.05. Tumors treated with 2 x 10^5 and 5 x 10^4 units HulFN-α(Ly) were significantly smaller than were control tumors shown in Chart 1C (p < 0.01 and < 0.025, respectively). No evidence of systemic toxicity was seen at any dose of IFN. For further experiments to study interactions between these agents, a dose of each was chosen that was not toxic, inhibited tumor size index after 35 days of therapy compared with control values, but did not cause regression.

Combination of IFN and CY. The data in Chart 2 show the effect of combining 0.25 mg CY given once a week 3 weeks in every 4 with varying doses of HulFN-α(Ly) given daily. With the highest dose of HulFN-α(Ly), 2 x 10^4 units, or CY therapy alone, tumor growth inhibition was seen, but no regression or tumor stasis was noted. However, when the 2 therapies were combined, the tumors in all mice failed to grow and regressed completely in 3 of 4 mice. With the 2 lower doses of HulFN-α(Ly) (5 x 10^4 and 2 x 10^4 units daily), increased activity was also seen when IFN was combined with CY and, even where there was no demonstrable effect of the IFN alone on the tumor (see Chart 2C), the combination of IFN and CY induced tumor stasis. At 35 days, the difference between control tumor sizes and all other therapy groups apart from 5 x 10^4 and 2 x 10^4 units HulFN-α(Ly) was statistically significant (p < 0.01 or p < 0.001). Also, in most instances, the tumor size indices in the single-
therapy groups were statistically larger than the indices obtained with combined therapy. At 55 days, results obtained with 2 x 10^6 units HuIFN-α(Ly) plus 0.25 mg CY were significantly better than either the CY alone (p < 0.01) or the IFN alone (p < 0.01). At 43 days, mice treated with 5 x 10^6 units HuIFN-α(Ly) plus 0.25 mg CY had significantly smaller tumors than did those treated with IFN alone (p < 0.01) or CY alone (p < 0.01), and mice treated with 2 x 10^6 units HuIFN-α(Ly) plus 0.25 mg CY had significantly smaller tumors than did those treated with IFN alone (p < 0.01).

The duration and reversibility of the effect of this combination therapy are shown by data in Table 1 and were dependent on the dose of HuIFN-α(Ly). Mice were killed when their tumors reached a size index of >2. At the highest dose of IFN, 3 of 4 mice receiving IFN-CY survived over 150 days without their tumors reaching >2. With 5 x 10^6 units HuIFN-α(Ly) and CY, in 3 of 4 mice tumors took 50 days after the end of therapy to reach a tumor size index of >2, whereas the control tumor grew to a tumor size index of 3.1 in 35 days from the start of the experiment.

Bone marrow toxicity of this combination in the mice was measured by determining peripheral blood WBC during therapy. The mean WBC of each group of mice was not significantly affected by either agent alone or a combination of the 2. For instance, in one experiment at the end of 39 days of therapy, control WBC were 7.63 ± 1.3 x 10^6/ml; and in the IFN-CY group, they were 7.2 ± 2 x 10^6/ml.

Experiments were also carried out to see if giving the IFN and CY sequentially was of benefit. Either 2 x 10^6 units IFN daily or 0.25 mg CY were administered to the mice for 28 days of a 56-day experiment, and then the therapy was reversed. While stronger antitumor effects were seen when 28 days of therapy with either agent was followed with 28 days of therapy with the other agent, as compared to 28 or 56 days of therapy with either agent alone, the interaction was not as strong as when the 2 agents were used concurrently, although the difference between these 2 groups was not statistically significant. Chart 3 shows an example of such an experiment in which CY was given before IFN. At 35 days, both the CY-IFN combined or CY-IFN sequential tumors were significantly smaller than were control tumors (p < 0.001).

Combination of IFN and ADR. Chart 4 shows results from experiments with 2 doses of ADR, 0.04 and 0.02 mg given once weekly, and 2 x 10^6 units IFN given daily. In both experiments, the combination of IFN with ADR gave better results than did either agent alone, with tumor regression in all mice receiving the combination with the highest dose of ADR and tumor stasis in mice given the lower dose. In Chart 4A, at 45 days, all treatment groups had tumors significantly smaller than the control tumors (p < 0.001, < 0.01, and < 0.001 for HuIFN-α(Ly), ADR, and the combination, respectively. At 45 and 56 days, the tumor sizes in the IFN- and ADR-alone groups were significantly larger than in the combined groups (At 45 days: IFN versus ADR plus IFN, p < 0.05; ADR versus ADR plus IFN, p < 0.025. At 56 days: IFN versus ADR plus IFN, p < 0.01; ADR versus ADR plus IFN, p < 0.05.). In Chart 4B, at 36 days, all treatment groups had tumor size indices significantly different from those of the control groups (data not shown) and, as in Chart 4A, the tumor sizes in the IFN- or ADR-alone groups were significantly larger than in the combined groups (IFN versus IFN plus ADR, p < 0.05; ADR versus IFN plus ADR, p < 0.01). The combination of HuIFN-α(Ly) and ADR had no bone marrow toxicity as measured by peripheral blood WBC. For instance, in one experiment at the end of 38 days, therapy control WBC were 7.7 ± 0.7 (S.D.) x 10^6 cells/ml, and IFN-ADR group WBC were 5.8 ± 2 x 10^6 cells/ml. Mice treated with IFN-ADR had a longer survival than did the other groups. In a typical experiment, 2 of 4 IFN-ADR-treated mice survived 260 days posttherapy and still remained tumor.

Table 1
Survival posttherapy of mice receiving HuIFN-α(Ly)-CY combination therapy

<table>
<thead>
<tr>
<th>CY (mg)</th>
<th>IFN (units)</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>2 x 10^6</td>
<td>187</td>
<td>152</td>
<td>359</td>
<td>80</td>
</tr>
<tr>
<td>0.25</td>
<td>5 x 10^6</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>152</td>
</tr>
<tr>
<td>0.25</td>
<td>2 x 10^6</td>
<td>29</td>
<td>29</td>
<td>50</td>
<td>152</td>
</tr>
</tbody>
</table>

a Killed 187 days posttherapy due to liver tumor of murine karyotype; no xenografted tumor visible.

b Killed 153 days posttherapy due to severe eczema. Xenografted tumor was 0.05 x 0.05 cm.

c Mouse still surviving.
free. The other 2 mice were killed at 62 and 83 days posttherapy when their tumors reached a size index of 2 or >2. The IFN-treated mice survived for 30 days (3 of 4) and 54 days (1 of 4) posttherapy, and 3 of 4 of the ADR-treated mice were killed 30 days posttherapy and 1 of 4 were killed at 217 days.

Comparison of Combinations of Drug plus IFN or Drug plus Drug. In the experiment shown in Chart 5, the effects of combinations of ADR and CY were compared to those of drug-IFN combinations in a 35-day experiment. A combination of ADR and CY was no better than either drug combined with IFN; in fact, the INF-CY therapy gave better results than did ADR-CY (p < 0.05). Complete regressions were seen in 2 of 4 mice in both the CY-IFN and CY-ADR-IFN groups.

DISCUSSION

In this paper, we have shown that combination of human IFN with suboptimal doses of ADR or CY greatly increases their antitumor activity on human tumor xenografts. A combination of agents that is more effective than is expected from the effectiveness of its constituents is said to show a positive interaction or synergy (6), but because the majority of dose-response curves from biologically active agents are not linear, it is difficult to define such interactions between 2 agents. Ideally, we should produce full dose-response curves of each agent and analyze the combined response by the isobologram method (6), but this approach would require more tumor-bearing mice than we are able to generate. However, we have been able to use our limited information to carry out the algebraic calculations of Berenbaum (6) of which the isobologram is the geometric equivalent. In these calculations, the doses of 2 agents, A and B, producing a specified effect (\(A_c\) and \(B_c\)) are divided into the dose of the 2 agents that produce the same effect when combined (\(A_c\) and \(B_c\)), i.e.,

\[
\frac{A_c}{A} + \frac{B_c}{B} = 1 \quad \text{for zero interaction}
\]

\[
\frac{A_c}{A} + \frac{B_c}{B} < 1 \quad \text{for synergy}
\]

\[
\frac{A_c}{A} + \frac{B_c}{B} > 1 \quad \text{for antagonism}
\]

Using the data from Chart 2 and other data from our limited dose-response curves, we obtain results such as 0.38 and 0.23 for this equation for IFN-CY combinations and 0.75 and 0.7 for IFN-ADR combinations. Thus, we conclude that the interactions between IFN and CY or ADR are positive or synergistic. Also, unequivocal evidence for positive interactions can be obtained when one of the agents in the combination has no effect, and this can be seen in Chart 2C where the dose of IFN used (2 x 10^4 units) has no effect on tumor size index but strongly increases the effect of CY in a combination.

Positive interactions between murine IFNs and chemotherapy have been reported previously. For instance, strong effects of IFN and CY were seen in mice with AKR lymphoma (12) and C1300 neuroblastoma (22), and a combination of IFN and 1,3-bis(2-chloro-ethyl)-1-nitrosourea was curative in systemic murine
leukemia (7). However, IFN failed to enhance the response of L1210 leukemia to 6-mercaptopurine, ADR, 1-β-o-arabinofuranesylcytosine or CY although some enhancement of the methotrexate response was seen (18), and rat IFN interfered with the beneficial effect of CY on a rat liposarcoma (14).

It is probable that interactions between IFNs and chemotherapeutic agents are complex. There may be direct interactions between the drugs and IFN on tumor cells or indirect effects of the IFN on drug metabolism. Concerning direct effects on the tumor cells, IFNs are known to lengthen all phases of the cell cycle (2, 4, 20) and thus could render cells more sensitive to both cycle- and phase-specific drugs by prolonging the time during which they are sensitive to the lethal effects of the drug. In an attempt to elucidate this, we are currently studying the cell cycle distribution of the nude mouse tumor treated with drugs and IFN. However, indirect effects of IFNs on drug metabolism may also be important when homologous IFNs are used in therapy. It is well documented that IFNs inhibit the cytochrome P-450 system in the liver (17) which is particularly important in the activation of CY to its alkylating agent metabolite, phosphoramide mustard (10). Also, if IFN affects other drug-metabolizing enzymes, for instance those involved in the breakdown and detoxification of chemotherapeutic agents, this could result in a prolonged exosure to the drug. The finding that rat IFN interfered with the antitumor effects of CY on a rat liposarcoma (14) and that mouse IFN protected mice from lethal doses of 5-fluorouracil (19) are evidence for the importance of such indirect actions.

The nude mouse human tumor xenograft is an ideal system in which to dissociate the direct and indirect antitumor action of IFN-drug combinations; as we have shown previously, human IFN has no effect on the nude mouse tissues in terms of levels of the IFN-induced enzyme, 2′-5′ oligoadenylate synthetase, and natural killer cells (1). Thus, the interactions observed here are most likely to be due to direct effects of drug and IFN on the tumor cell, and when the IFN is homologous these interactions may be altered. The addition of mouse IFN into the human IFN-drug combinations will allow us to dissociate between such direct effects on the tumor and indirect effects of IFN on the drug metabolism by the murine tissues; such experiments are now under way. Preliminary data indicate that equal amounts of mouse IFN (in terms of biological units) have no significant effect on the HuILN-α(Ly)-CY or the HuILN-α(Ly)-ADR combination, either in altering the activity on the human tumor or in increasing the toxicity of the therapy.3

We conclude from the data presented here that interactions between IFNs and cytotoxic drugs may have therapeutic potential but are complex and may be dependent on dose duration of exposure to each agent and the effects of IFN on the metabolism of the drug. It will be important to extend these studies to other tumor xenografts and drugs.

3 F. R. Balkwill, unpublished data.

ACKNOWLEDGMENTS

We wish to thank Dr. K. H. Fantes, Welcome Research Laboratories, United Kingdom, for the purified Namaiwa IFN; Dorota Griffin and Heather Band for assisting in cloning this IFN; and Vivienne Freedman for karyotyping the tumors.

REFERENCES


CANCER RESEARCH VOL. 44
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